RESTRICTION SITE MAPPING OF THE CHLOROPLAST DNA INVERTED REPEAT: A MOLECULAR PHYLOGENY OF THE ASTERIDAE[1]

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ABSTRACT

By focusing exclusively on the highly conserved inverted repeat region of the chloroplast genome, we extend comparative restriction site mapping to greater evolutionary depths than those to which it has been applied previously. A cladistic analysis of inverted repeat restriction site data is presented in order to enhance understanding of relationships within the Asteridae and to test the possible monophyly of the Asteridae. A total of 114 species, representing 33 families of Asteridae and eight families of Rosidae and Dilleniidae (sensu Cronquist) was examined, of which 99 species exhibited restriction maps of sufficient colinearity to be included in the phylogenetic analysis. Analysis with four restriction endonucleases identified a total of 77 restriction sites, 55 of which were phylogenetically informative. Parsimony analysis identified six major groups that broadly correspond to traditionally recognized orders in the Asteridae: Asterales, Boraginales, Dipsacales, Gentianales, Scrophulariales plus Lamiales, and Solanales. The results further suggest that the Asteridae, as traditionally circumscribed, are not monophyletic. The Apiaceae, Araliaceae, Cornaceae, Hydrangeaceae, Loasaceae, and possibly the Fouquieriaceae, all placed previously by Cronquist in subclasses Dilleniidae and Rosidae, should be included in a broadly defined Asteridae. The Cornaceae, Hydrangeaceae and Loasaceae appear closely related. Unexpected results include a probable sister-group relationship between the Apiales and Dipsacales, and the placement of the Menyanthaceae in the Asterales. Familial relationships within several orders and interordinal relationships remain poorly resolved.

The Asteridae, comprising about a third of all dicot species, are the second largest subclass of dicots and are thought to be of relatively recent origin compared to other dicot subclasses (Cronquist, 1981). Most members are characterized by derived features of floral morphology (e.g., sympetalous flowers), embryology, and chemistry. Although the concept of Asteridae can be traced back to the late seventeenth and early eighteenth centuries (de Jussieu, 1789; de Candolle, 1813), the present circumscription of this group as a subclass was described initially by Takhtajan (1964). Phylogenetic relationships within recent classifications of the subclass (sensu Cronquist, 1981, and Takhtajan, 1980) have remained unclear despite intensive morphological, anatomical, and phytochemical analyses. Substantial disagreement exists regarding interfamilial and interordinal relationships, with many genera and families of uncertain familial or ordinal placement. Moreover, the monophyly of the subclass itself has been disputed (Dahlgren, 1983; Thorne, 1983; Takhtajan, 1987). To date, very few cladistic studies that treat taxa above the family level have been put forth for the Asteridae (Cantino, 1982; Lu, 1990; Olmstead et al., 1990; Michaels et al., in prep.; other studies in this volume).

Although phylogenetic analysis of restriction site variation in chloroplast genomes has been used to construct explicit hypotheses of relationships in a number of plant groups (reviewed in Palmer, 1987; Palmer et al., 1988), it has never been applied at taxonomic levels higher than the rank of family. In fact, restriction site analysis has generally been viewed as inappropriate at these levels. As phylogenetic distance and molecular divergence increase, so does the proportion of homoplastic site changes. Additionally, the accumulation of inser-

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tions and deletions prevents alignment of restriction maps and assessment of site homology. Higher-level relationships have been studied previously by chloroplast gene sequencing (e.g., Soltis et al., 1990b; Olmstead et al., 1992) and by the distribution of major structural rearrangements in the chloroplast genome (e.g., Jansen & Palmer, 1987; Downie et al., 1991).

While investigating chloroplast genome structural variation in a wide array of angiosperms (Downie & Palmer, 1991), we noticed that the chloroplast DNA (cpDNA) of many species possessed restriction fragments in their large inverted repeat (IR) that were similar in size. In a typical angiosperm chloroplast genome of 150 kb, each of the two IR copies is about 25 kb in size. The remarkable conservation in size of IR restriction fragments across most dicot lineages lead us to believe that it might be possible to analyze restriction sites in this region for phylogenetic purposes. Preliminary mapping studies revealed that many restriction sites were indeed conserved across major lineages of Asteridae.

The first evidence that IR sequences in cpDNA have lower mutation rates than single-copy sequences came from restriction site analyses at the intrafamilial level (Palmer & Zamir, 1982; Palmer et al., 1983a, b; Clegg et al., 1984; Sytsma & Schaal, 1985; Jansen & Palmer, 1987). A detailed examination of gene sequences confirmed this inference and demonstrated that rates of nucleotide substitution at silent (synonymous) sites and in noncoding sequences in the IR are 4–6 times lower than those in the large and small single-copy regions (Wolfe et al., 1987; K. Wolfe, unpublished). The rate of insertions and deletions also appears to be suppressed in the IR compared to single-copy regions (Doebley et al., 1987; Jansen & Palmer, 1988; Schilling & Jansen, 1989; Wallace & Jansen, 1990; Soltis et al., 1990b; K. Wolfe, C. Morden & J. Palmer, unpublished). The evolutionary processes and molecular mechanisms responsible for mutation rates in the IR are not understood.

Here we present results from a cladistic analysis of cpDNA IR restriction site variation within the Asteridae. Since the study presented here was not designed to sample restriction site changes comprehensively, but rather to look for phylogenetically useful structural changes, only four restriction enzymes were used instead of the 10–25 that typify such studies. Nevertheless, a substantial number of restriction site mutations was obtained in this pilot study. Our goal in presenting this study is twofold: (1) to demonstrate, by focusing exclusively on the highly conserved IR region of the chloroplast chromosome, that comparative restriction site mapping can be extended to taxonomic levels higher than previously considered possible, and (2) to formulate more precise hypotheses about relationships among the diverse clades comprising the Asteridae and to test their monophyly and origins relative to putatively ancestral groups in the Rosidae and Dilleniidae.

Materials and Methods

Material of 114 species, representing 103 species of Asteridae, 2 species of Dilleniidae, and 9 species of Rosidae (sensu Cronquist, 1981), was field collected or obtained from various sources, either as fresh leaf material or DNA. A list of sources and voucher information for all taxa examined herein is available upon request. The 114 species represent 33 of 49 families of Asteridae (sensu Cronquist), two families of Dilleniidae, and six families of Rosidae (Table 1). The remaining families treated by Cronquist in subclass Asteridae were omitted from the analysis because material was unavailable for study. The isolation of cpDNA or total cellular DNA from leaf material was accomplished using the sucrose gradient technique of Palmer (1986) or the modified CTAB procedure of Doyle & Doyle (1987), respectively. All cpDNAs and most total DNAs were further purified by centrifugation in cesium chloride/ethidium bromide gradients.

All DNAs were digested singly with each of four restriction endonucleases: *Bam*HI, *Bgl*II, *Eco*RV, and *Hin*dIII. DNA digests for 75 of the 114 species listed in Table 1 were separated electrophoretically in 1.0% agarose gels in which the bromophenol blue dye marker was run 6 cm. In this way four 20-cm-wide filters could be placed on a standard 20 × 25-cm x-ray film. For the remaining 39 species, which were analyzed at a later date, the dye marker was run 12 cm. The resulting cpDNA fragments were bidirectionally transferred to nylon filters (Zetabind, AMF Cuno), and visualized by filter hybridizations using ^{32}P-labeled probes (described below) and autoradiography (Palmer, 1986; Palmer et al., 1988; Downie & Palmer, 1991). Size markers used were equimolar mixtures of phage lambda DNA digested with *Eco*RI and *Hin*dIII and with *Hin*dIII alone. Filters were washed in 2× SSC, 0.5% SDS twice for 5–10 minutes at room temperature and 2–3 times for 60 minutes at 65°C prior to autoradiography. Twenty-six subclones of a *Nicotiana tabacum* cpDNA library (Sugiura et al., 1986; Fig. 1) were used as hybridization probes. These probes span the entire *N. tabacum* cpDNA

TABLE 1. Taxa scored for inverted repeat restriction site variation. System of classification follows that of Cronquist (1981). Asterisks denote plants that are not included in the cladistic analysis because their chloroplast genomes are rearranged or extremely divergent in base sequence relative to *Nicotiana tabacum*.

DILLENIIDAE
- Violales
  - Fouquieriaceae
    - *Fouquieria splendens*
  - Loasaceae
    - *Eucnide hirta*

ROSIDAE
- Apiales
  - Apiaceae
    - *Coriandrum sativum*
  - Araliaceae
    - *Hedera helix*
    - *Trevesia sundaica*
- Cornales
  - Cornaceae
    - *Aucuba japonica*
    - *Cornus mas*
    - *Cornus kousa*
- Rosales
  - Grossulariaceae
    - *Ribes americanum*
  - Hydrangeaceae
    - *Hydrangea* sp.
  - Rosaceae
    - *Spiraea nipponica*

ASTERIDAE
- Asterales
  - Asteraceae
    - *Barnadesia caryophylla*
    - *Lactuca sativa*
- Callitrichales
  - Callitrichaceae
    - *Callitriche heterophylla*
- Calycerales
  - Calyceraceae
    - *Boopis graminea*
    - *Gamocarpha poeppigii*
- Campanulales
  - Campanulaceae
    - *Campanula garganica**
    - *Campanula ramosa**
    - *Hippobroma longiflora**
    - *Jasione montana**
    - *Lobelia erinus**
    - *Lobelia laxiflora**
    - *Monopsis lutea**
    - *Platycodon grandiflorus**
    - *Sclerotheca jayorum**
  - Goodeniaceae
    - *Goodenia ovata**
    - *Scaevola taccada**
- Dipsacales
  - Caprifoliaceae
    - *Kolkwitzia amabilis*
    - *Lonicera subsessilis*
    - *Sambucus canadensis*
    - *Symphoricarpos albus*
    - *Viburnum acerifolium*
    - *Weigela hortensis*
  - Dipsacaceae
    - *Cephalaria leucantha*
    - *Dipsacus sativus*
    - *Scabiosa ochroleuca*
  - Valerianaceae
    - *Valeriana* sp.
- Gentianales
  - Apocynaceae
    - *Apocynum cannabinum*
    - *Acokanthera oblongifolia*
    - *Ochrosia eliptica*
    - *Prestonia acutifolia*
    - *Vinca minor*
  - Ascelpiadaceae
    - *Asclepias curassavica*
    - *Asclepias exaltata*
    - *Periploca sepium*
  - Gentianaceae
    - *Exacum affine*
    - *Gentiana dahurica*
    - *Lisianthus skinneri*
    - *Obolaria virginica*
  - Loganiaceae
    - *Fagraea zeylanica*
    - *Spigelia marilandica*
    - *Strychnos spinosa*
- Lamiales
  - Boraginaceae
    - *Borago officinalis*
    - *Heliotropium arborescens*
    - *Mertensia virginica*
  - Lamiaceae
    - *Comanthosphace stellipila*
    - *Melissa officinalis*
    - *Pogostemon patchulii*
    - *Prasium majus*
    - *Prostanthera nivea*
    - *Salvia divinorum*
    - *Scutellaria bolanderi*
    - *Stachys officinalis*
    - *Teucrium canadense*
  - Verbenaceae
    - *Callicarpa dichotoma*
    - *Caryopteris clandonensis*
    - *Clerodendrum fragrans*
    - *Clerodendrum ugandense*
    - *Phyla scaberrima*
    - *Phryma leptostachya*
    - *Premna japonica*
    - *Verbena bonariensis*
- Rubiales
  - Rubiaceae
    - *Pentas lanceolata*

TABLE 1. Continued.

Scrophulariales
  Acanthaceae
    Graptophyllum pictum
    Justicia carnea
    Pachystachys lutea
  Bignoniaceae
    Campsis radicans
    Catalpa bignonioides
    Clytostoma callistegioides
  Buddlejaceae
    Buddleja davidii
  Gesneriaceae
    Alsobia dianthiflora
    Nematanthus hirsutus
  Globulariaceae
    Globularia salicinus
  Myoporaceae
    Eremophila maculata
    Myoporum sandwicense
  Oleaceae
    Forysthia sp.
    Ligustrum sinensis
    Syringa vulgaris
  Orobanchaceae
    *Conopholis americana**
    *Epifagus virginiana**
  Pedaliaceae
    Proboscidea louisianica
    Sesamum indicum
  Scrophulariaceae
    Antirrhinum majus
    Digitalis parviflorum
    Paulownia tomentosa
    *Striga asiatica**
    Verbascum thapsus
Solanales
  Convolvulaceae
    Calonyction aculeatum
    Convolvulus tricolor
    Ipomoea pes-caprae
  Cuscutaceae
    Cuscuta sp.*
  Hydrophyllaceae
    Eriodictyon californica
    Hydrophyllum virginiana
  Menyanthaceae
    Fauria crista-galli
    Menyanthes trifoliata
    Nymphoides peltata
    Villarsia calthifolia
  Nolanaceae
    Nolana spathulata
  Polemoniaceae
    Phlox 'Pinafore Pink'
    Polemonium reptans
  Solanaceae
    Iochroma cyaneum
    Nicotiana tabacum
    Schizanthus pinnatus
    Solandra grandiflora

IR and adjacent portions of the single-copy regions, and range in size from 0.2 to 3.3 kb (averaging approximately 1 kb).

Unambiguous restriction site maps for each of the four enzymes were constructed for *Nicotiana tabacum* by computer analysis of its completely known cpDNA sequence (Shinozaki et al., 1986; Fig. 1). Because many restriction sites and fragment sizes among the taxa examined coincided with those known in *N. tabacum*, mapping efforts were greatly facilitated by scoring our data against these maps. The inclusion of one lane of *N. tabacum* cpDNA on all filters permitted a comparison between the expected size of a particular fragment in *N. tabacum* (as ascertained from the computer-generated map) and the observed size in other taxa.

Parsimony analyses of the restriction site data were conducted using PAUP version 3.0k- (Swofford, 1990) on a Macintosh IIfx computer. All three branch-swapping algorithms and the three sequences of taxon addition (simple, closest, and random) used by PAUP were employed in an attempt to find the most parsimonious trees. The data matrix is available upon request.

To assess the circumscription and possible monophyly of the Asteridae, a number of outgroups were chosen from putatively related taxa in Rosidae and Dilleniidae sensu Cronquist. These taxa included representatives from the Apiaceae, Araliaceae, Cornaceae, Fouquieriaceae, Grossulariaceae, Hydrangeaceae, Loasaceae, and Rosaceae (Table 1). Among current classifications, a consensus exists favoring a "Rosalean" ancestry for the Asteridae (e.g., Cronquist, 1981; Takhtajan, 1987); however, the exact boundaries between the Rosidae and Asteridae are subject to some dispute. Results from a concurrent phylogenetic analysis of *rbc*L sequence data (Olmstead et al., 1992) indicate that the Apiaceae, Araliaceae, Cornaceae, and Hydrangeaceae should all be included in a broadly defined Asteridae. Other evidence suggests the same for the Loasaceae (Takhtajan, 1980) and Fouquieriaceae (Thorne, 1977). Consequently, *Spiraea nipponica* (Rosaceae, Rosidae) was ultimately

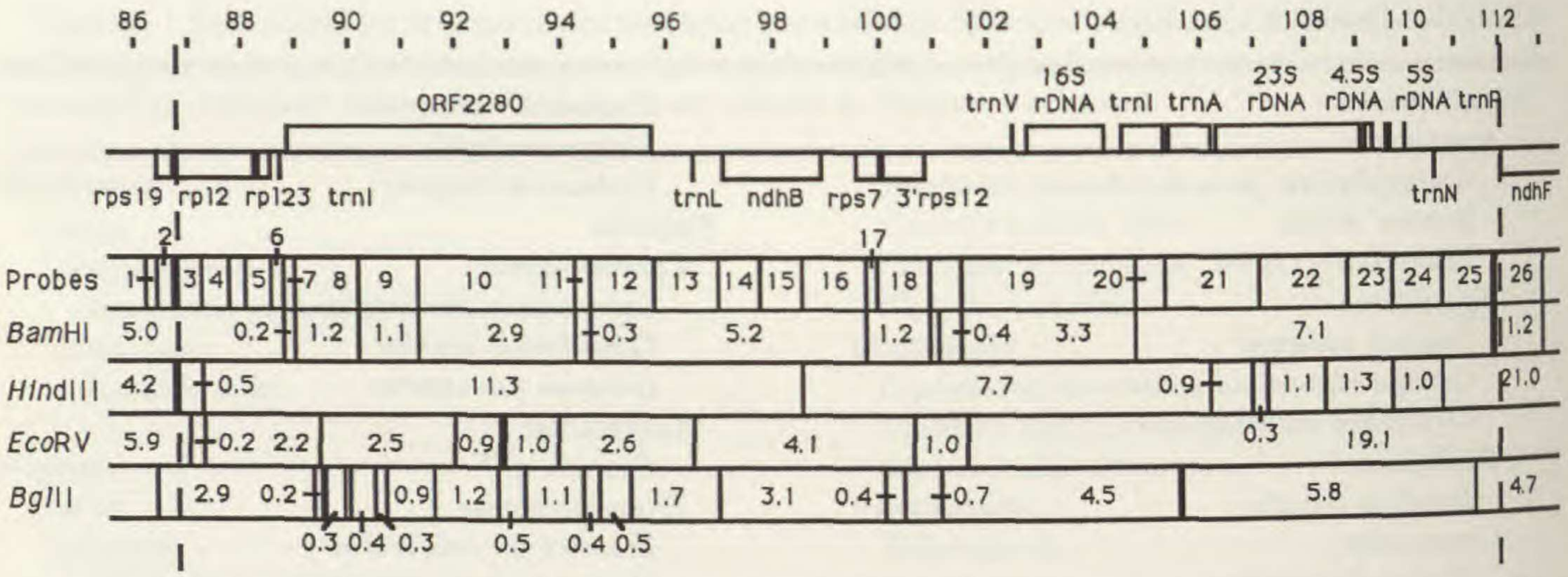


FIGURE 1. Gene and restriction site maps of the inverted repeat (IR) and adjacent single-copy regions of *Nicotiana tabacum* cpDNA. Cleavage sites, gene locations, and sequence coordinates in kb (scale on top) are modified from Shinozaki et al. (1986). Restriction fragment sizes are indicated in kb. The subclones used as hybridization probes are numbered from 1 to 26. Probe 3 spans the junction (sequence coordinate 86685) between the IR and the large single-copy region; probe 26 spans the junction (sequence coordinate 112023) between the IR and the small single-copy region. The boundaries of the *N. tabacum* IR are indicated by vertical dashed lines. Restriction site data from 114 species of dicotyledonous plants were scored against these maps.

chosen as the outgroup in this analysis, because the Rosaceae are clearly excluded from the Asteridae in all modern systems of classification. The trees computed by PAUP were rooted by positioning the root along the branch connecting *Spiraea* to the rest of the network (see simultaneous resolution procedure, Maddison et al., 1984).

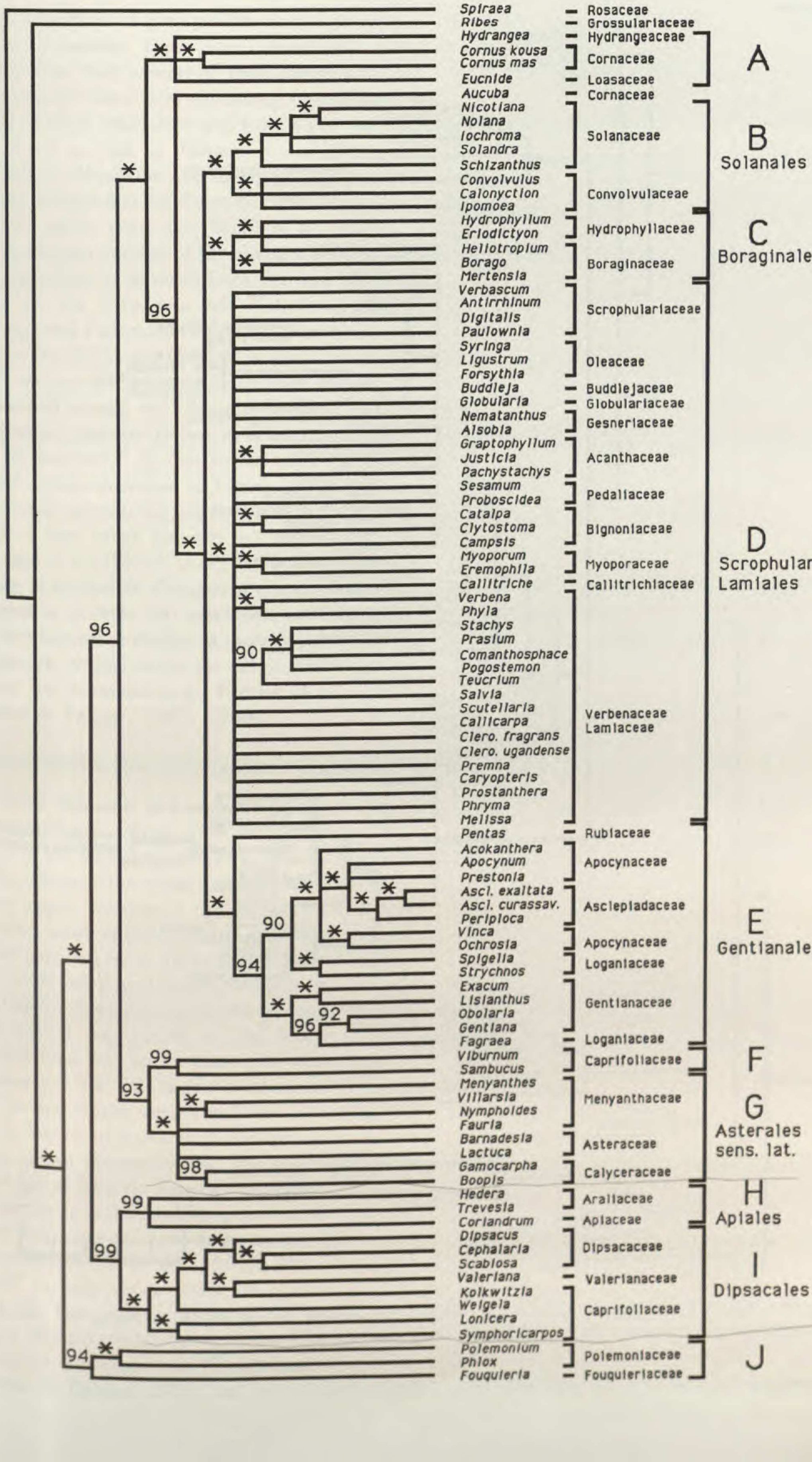
RESULTS AND DISCUSSION

(1) RESTRICTION SITE VARIATION

Restriction site maps of IR sequences for each of 114 species (Table 1) were constructed for four enzymes using 26 hybridization probes from *Nicotiana tabacum* (Fig. 1). The maps reveal that the IR of 99 of these cpDNAs is both colinear in gene arrangement and readily aligned with that of *N. tabacum* (and, thereby, also with the IR of the majority of angiosperms so far examined). In contrast, at the interfamilial level, little or no alignment of restriction sites was possible in adjacent single-copy regions. Restriction site maps of the IR for 15 species (Table 1) could not be aligned with the computer-generated base maps of *N. tabacum*. Consequently, these taxa were excluded from the cladistic analysis. Excluded were two parasitic asterid genera, *Conopholis* (Orobanchaceae) and *Striga* (Scrophulariaceae), which have each lost one entire segment of their cpDNA IR. Increased sequence divergence in those regions that are contained within an IR in most angiosperms made the assessment of site homology difficult. Except for *Conopholis* and *Striga*, variation in size of the IR was minimal and did not confound interpretation. The 13 other excluded species, from the Campanulaceae (including Lobeliaceae), Goodeniaceae, Cuscutaceae, and Orobanchaceae (Table 1), all possessed a large IR but were otherwise too rearranged and divergent in sequence relative to *N. tabacum* to be included in the analysis.

Comparison of the 99 alignable restriction site maps, representing 37 families of dicots, revealed low levels of restriction site divergence. Fifty-five (71.5%) of the 77 restriction sites identified among 99 taxa were shared by two or more taxa and were thus informative for phylogenetic analysis; 17 (22%) of the remaining sites were unvarying, and five (6.5%) were unique to individual taxa and, therefore, provided no phylogenetic information. The 77 restriction sites examined represent 462 bp or 1.8% of the entire IR and 0.3% of the entire chloroplast genome. The occurrence of many invariant restriction sites and of readily identifiable homologous sites across 37 families belonging to

FIGURE 2. Majority-rule consensus tree consistent with 90% of 5,000 equally parsimonious 159-step trees derived from Wagner parsimony. Asterisks denote clades that are consistent with 100% of the equally parsimonious trees. Complete names of all taxa are provided in Table 1. Taxa are divided into ten groups (A to J) and are discussed in the text. Familial designations follow Cronquist (1981).


Spiraea
Ribes
Hydrangea
Cornus kousa
Cornus mas
Eucnide
Aucuba
Nicotiana
Nolana
Iochroma
Solandra
Schizanthus
Convolvulus
Calonyction
Ipomoea
Hydrophyllum
Eriodictyon
Heliotropium
Borago
Mertensia
Verbascum
Antirrhinum
Digitalis
Paulownia
Syringa
Ligustrum
Forsythia
Buddleja
Globularia
Nematanthus
Alsobia
Graptophyllum
Justicia
Pachystachys
Sesamum
Proboscidea
Catalpa
Clytostoma
Campsis
Myoporum
Eremophila
Callitriche
Verbena
Phyla
Stachys
Prasium
Comanthosphace
Pogostemon
Teucrium
Salvia
Scutellaria
Callicarpa
Clero. fragrans
Clero. ugandense
Premna
Caryopteris
Prostanthera
Phryma
Melissa
Pentas
Acokanthera
Apocynum
Prestonia
Ascl. exaltata
Ascl. curassav.
Periploca
Vinca
Ochrosia
Spigelia
Strychnos
Exacum
Lisianthus
Obolaria
Gentiana
Fagraea
Viburnum
Sambucus
Menyanthes
Villarsia
Nymphoides
Fauria
Barnadesia
Lactuca
Gamocarpha
Boopis
Hedera
Trevesia
Coriandrum
Dipsacus
Cephalaria
Scabiosa
Valeriana
Kolkwitzia
Weigela
Lonicera
Symphoricarpos
Polemonium
Phlox
Fouquieria
Rosaceae
Grossulariaceae
Hydrangeaceae
Cornaceae
Loasaceae
Cornaceae
Solanaceae
Convolvulaceae
Hydrophyllaceae
Boraginaceae
Scrophulariaceae
Oleaceae
Buddlejaceae
Globulariaceae
Gesneriaceae
Acanthaceae
Pedaliaceae
Bignoniaceae
Myoporaceae
Callitrichaceae
Verbenaceae
Lamiaceae
Rubiaceae
Apocynaceae
Asclepiadaceae
Apocynaceae
Loganiaceae
Gentianaceae
Loganiaceae
Caprifoliaceae
Menyanthaceae
Asteraceae
Calyceraceae
Araliaceae
Apiaceae
Dipsacaceae
Valerianaceae
Caprifoliaceae
Polemoniaceae
Fouquieriaceae
A
B
Solanales
C
Boraginales
D
Scrophulariales/
Lamiales
E
Gentianales
F
G
Asterales
sens. lat.
H
Apiales
I
Dipsacales
J
96
96
90
94
90
92
96
99
93
98
99
99
94

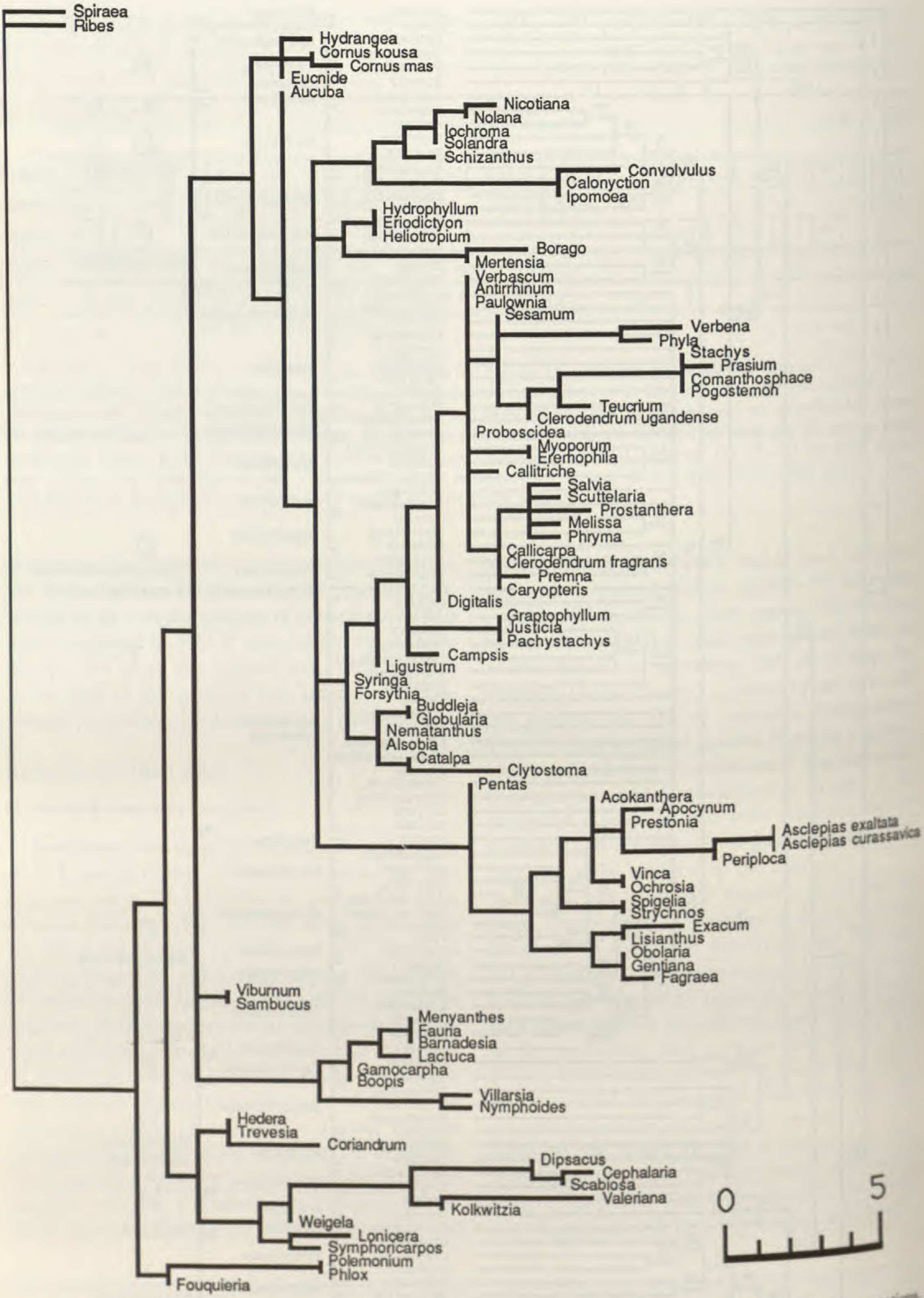


FIGURE 3. One of 5,000 equally parsimonious Wagner trees based on cpDNA IR restriction site mutations. Branch lengths are proportional to the number of inferred mutations.

three subclasses of dicotyledons (sensu Cronquist, 1981) is notable. Even more remarkable is the observation that several of these restriction sites were found to be conserved among representatives from all dicot subclasses and from some monocot subclasses as well (S. Downie & J. Palmer, unpublished). Monocots and dicots represent angiosperm lineages that may have diverged on the order of 200 million years ago (Wolfe et al., 1989).

The largest number of site changes between any pair of DNAs as inferred from the tree in Figure 3 is 21, for *Nicotiana* and *Valeriana* and for *Borago* and *Valeriana* (Table 3). In contrast, several of the DNAs are identical in all 77 restriction sites compared. Sequence divergence values were calculated among the outgroup species, *Spiraea nipponica*, and one (or two, as in the case of Group D) representative species within each of the 10 major groups identified in Figure 2. Estimates of nucleotide substitution per site ($100 \times P$) between pairs of taxa range between 0.7 and 8.5 with an average of 4.5 (Brown et al., 1979; Table 3). This range of nucleotide divergence among species belonging to at least two subclasses and ten orders of dicotyledons is similar to those reported for intergeneric studies where the entire chloroplast genome was examined (e.g., Palmer et al., 1983a; Jansen & Palmer, 1987, 1988).

(2) RESTRICTION FRAGMENT LENGTH VARIATION

Three deletions, one insertion, and one nonpolarizable length variant were detected in cpDNAs of 27 of the 99 species (Table 2). We have greatly underestimated the actual extent of restriction fragment length variation in the 99 species examined because most cpDNA length mutations (for the entire genome) are 1–10 bp in size (Palmer, 1985). We could not detect length variants smaller than 300 bp for those gels where the bromophenol blue dye marker was run 6 cm and length variants smaller than 200 bp for those gels where the dye marker was run 12 cm (see methods section).

The first length variant in Table 2 corresponds to the loss of an intron from the *rpl2* gene. It has been shown previously that the *rpl2* intron has been lost at least six times in the dicots, including at least twice in the Asteridae, once in the ancestral Convolvulaceae and once in the ancestral Menyanthaceae (Downie et al., 1991). The four other length variants occur within the gene ORF2280 (Fig. 1). This gene is absent in the grasses, and major deletions within the gene are known in several other nonasterid taxa (Hiratsuka et al., 1989; Downie & Palmer, 1991, and unpublished). The absence of any detectable length variation in intergenic spacers is surprising, as these comprise 23% of the *Nicotiana tabacum* IR and presumably could easily accommodate the disruptive effects of insertions and deletions. The rarity of length variation within the IR relative to single-copy sequences has been reported previously (Doebley et al., 1987; Jansen & Palmer, 1988; Schilling & Jansen, 1989; Wallace & Jansen, 1990; Soltis et al., 1990a; K. Wolfe, C. Morden & J. Palmer, unpublished).

Four of these length variants were shared by two or more taxa and, thus, are phylogenetically informative. Although length mutations were not used in the phylogenetic analysis, the phylogeny constructed from IR restriction site mutations (see below; Figs. 2 and 3) implies that the second, third, and fourth length mutations described in Table 2 have occurred independently eight, one, and three times, respectively. For those taxa possessing deletions, restriction sites in these regions were scored as missing data.

(3) PHYLOGENETIC ANALYSIS OF INVERTED REPEAT RESTRICTION SITE MUTATIONS

A phylogenetic analysis using Wagner parsimony and 99 taxa resulted in many shortest trees of 159 steps, with a consistency index (CI) of 0.36 (excluding uninformative characters). Sanderson & Donoghue (1989) found CI to be inversely correlated with the number of taxa included in an analysis. The largest data sets analyzed in their study included 65–68 taxa and had CI values of 0.32–0.37. Although there was no data set in their study comparable in number of taxa to ours, a CI value of 0.36 for 99 taxa appears to be higher than expected.

The exact number of minimum length trees could not be determined. PAUP found 9,000 159-step trees before the computer's memory was exhausted and the analysis terminated. Subsequently, the maximum number of trees saved by PAUP was arbitrarily set to 5,000, and from these a 90% majority rule consensus tree was derived (Fig. 2). Given the exploratory nature of this study, a 90% majority rule consensus tree was chosen over a strict consensus tree in order to offer greater resolution among the clades. However, it is stressed that the percentage values presented in Figure 2 do not measure the robustness of the clades, but rather indicate how many times particular groups of plants fall out together as monophyletic in the 5,000 shortest trees examined. The large number of taxa (99) relative to the number of informative characters (55) resulted in few synapomorphies

TABLE 2. Chloroplast DNA restriction-fragment length variation[a] in the inverted repeat region in Asteridae and related genera.

Probe[b]	Variation[c]	Size (bp)	Species
4	Deletion	600	Convolvulaceae
			Calonyction aculeatum
			Convolvulus tricolor
			Ipomoea pes-caprae
			Menyanthaceae
			Fauria crista-galli
			Menyanthes trifoliata
			Nymphoides peltata
			Villarsia calthifolia
8	Deletion	400	Apiaceae
			Coriandrum sativum
			Araliaceae
			Hedera helix
			Trevesia sundaica
			Asclepiadaceae
			Asclepias curassavica
			Asclepias exaltata
			Periploca sepium
			Bignoniaceae
			Catalpa bignonioides
			Clytostoma callistegioides
			Caprifoliaceae
			Kolkwitzia amabilis
			Lonicera subsessilis
			Symphoricarpos albus
			Weigela hortensis
			Convolvulaceae
			Calonyction aculeatum
			Convolvulus tricolor
			Ipomoea pes-caprae
			Dipsacaceae
			Cephalaria leucantha
			Dipsacus sativus
			Scabiosa ochroleuca
			Oleaceae
			Ligustrum sinensis
			Pedaliaceae
			Sesamum indicum
			Solanaceae
			Schizanthus pinnatus
			Valerianaceae
			Valeriana sp.
10	Insertion	500	Caprifoliaceae
			Kolkwitzia amabilis
			Lonicera subsessilis
			Symphoricarpos albus
			Weigela hortensis
			Dipsacaceae
			Cephalaria leucantha
			Dipsacus sativus
			Scabiosa ochroleuca
			Valerianaceae
			Valeriana sp.
12–13	Deletion	500	Bignoniaceae
			Clytostoma callistegioides

TABLE 2. Continued.

Probe[b]	Variation[c]	Size (bp)	Species
			Convolvulaceae
			Calonyction aculeatum
			Convolvulus tricolor
			Ipomoea pes-caprae
			Solanaceae
			Schizanthus pinnatus
12–13	Nonpolarizable	500	Rosaceae
			Spiraea nipponica

[a] Only length variants greater than 200–300 bp were detected (see text).
[b] See Figure 1 for map coordinates in kb and region of gene deletion/insertion.
[c] Restriction-fragment length variation relative to *Nicotiana tabacum*.

supporting each clade in the most parsimonious trees. The distribution of character support in one of these 5,000 equally parsimonious trees (chosen because of its similarity with the consensus tree) is illustrated in Figure 3, with nearly two-thirds of the nonterminal branches supported by only one character change. This, combined with the great length of time for the computer analyses, suggests that a bootstrap analysis (Felsenstein, 1985) to provide a quantitative measure of support for the clades identified in the consensus tree would be both inappropriate and impractical. Among 99 taxa and 77 restriction sites compared, the number of mutations for each site inferred from the tree in Figure 3 ranged from 0 to 8 with a mean of 2.1. Consequently, many of the single-length branches are characterized by homoplasious mutations. In spite of the low ratio of characters to taxa and the large number of equally parsimonious trees, a high degree of resolution is attained in some portions of the cladogram. The clades identified by an asterisk in Figure 2 were consistent with 100% of the equally parsimonious trees. The distribution of homoplasy is such that it effectively increases the number of characters supporting many branches.

(4) PHYLOGENETIC IMPLICATIONS OF CHLOROPLAST DNA MUTATIONS

Ten groups are identified that either coincide with orders recognized traditionally in the subclass or present novel relationships. These ten groups, identified from top to bottom in Figure 2, are: (A) a clade consisting of *Cornus*, Hydrangeaceae, and Loasaceae; (B) Solanales; (C) Boraginales; (D) Scrophulariales plus Lamiales; (E) Gentianales; (F) a clade consisting of *Viburnum* and *Sambucus*; (G) a clade consisting of Asterales, Calyceraceae, and Menyanthaceae; (H) Apiales; (I) Dipsacales (minus *Viburnum* and *Sambucus*); and (J) a basal

TABLE 3. Estimated nucleotide sequence divergence of the cpDNA IR among species of Asteridae and related genera. Complete names of species and their ordinal placement are presented in Table 1. The upper right portion of the matrix indicates the number of IR restriction site mutations between the two taxa as determined by direct pairwise comparisons. Pairwise nucleotide sequence divergence estimates are expressed by $100 \times p$ in the lower left portion of the matrix (Brown et al., 1979). The number of restriction sites examined for each species ranged from 37 to 48.

Species	1	2	3	4	5	6	7	8	9	10	11	12
1. *Spiraea*	—	8	16	10	11	11	17	9	12	10	17	7
2. *Hydrangea*	3.0	—	10	9	7	5	17	5	10	8	17	5
3. *Nicotiana*	6.4	4.4	—	13	7	5	19	11	16	12	21	11
4. *Borago*	4.1	3.0	4.7	—	10	8	18	11	16	14	21	12
5. *Verbascum*	4.4	2.6	2.7	4.3	—	2	16	8	13	9	16	6
6. *Syringa*	4.4	1.8	1.9	3.5	0.7	—	14	6	11	9	16	6
7. *Asclepias*	7.5	6.8	8.0	8.2	6.8	5.9	—	18	19	17	19	16
8. *Viburnum*	3.5	1.8	4.0	4.1	3.0	2.2	7.3	—	7	7	12	4
9. *Lactuca*	4.9	3.7	6.3	6.4	5.1	4.3	8.2	2.6	—	12	17	7
10. *Coriandrum*	3.3	2.3	3.2	6.6	2.8	2.8	6.6	1.9	4.1	—	13	7
11. *Valeriana*	6.9	6.3	8.2	8.4	7.1	6.3	8.5	4.5	6.8	4.4	—	14
12. *Fouquieria*	2.8	1.8	4.1	5.0	2.3	2.3	6.6	1.5	2.7	2.0	5.3	—

branch consisting of *Fouquieria* and Polemoniaceae.

The major clade comprising groups B, C, D, and E is consistent with the subclass Lamiidae as recognized by Takhtajan (1987), but with the exclusion of the Menyanthaceae and Loasaceae. For the most part, relationships among the ten groups are poorly resolved.

Monophyly of the Asteridae. Disagreement prevails as to whether or not the Asteridae are monophyletic. Cronquist (1981), Stebbins (1974), Takhtajan (1980), and Wagenitz (1977) consider the Asteridae to be a natural group, implying that they are monophyletic, whereas Philipson (1977), Dahlgren (1983), Throne (1983), and Takhtajan (1987) view the Asteridae (sensu Cronquist) as unnatural. Our results indicate that the Asteridae, as traditionally circumscribed, are not monophyletic. The Apiaceae, Araliaceae, Cornaceae, Hydrangeaceae, Loasaceae, and possibly the Fouquieriaceae, all traditionally placed in the Rosidae or Dilleniidae (e.g., Cronquist, 1981), should, in our view, be included within a broadly defined Asteridae. Additional molecular support for the inclusion of the Apiaceae, Araliaceae, Cornaceae, and Hydrangeaceae in Asteridae sens. lat. comes from *rbc*L sequence data (Olmstead et al., 1992, and unpublished). Olmstead et al. (1992) have shown that the origin and diversification of the Asteridae sens. lat. lie deep within the "higher" dicots, i.e., that the subclass is not of recent origin as argued by Cronquist (1981). Our results, using *Spiraea* as an outgroup, are generally in accord with those obtained from the *rbc*L analysis, in which representatives from the Magnoliidae were used as outgroups (Olmstead et al., 1992).

These results indicate that the subclass does not entirely correspond to its usual circumscription as a primarily sympetalous group. The occurrence of distinct petals in *Aucuba* (Cornaceae), the Apiales, and the clade consisting of *Cornus* (Cornaceae), *Hydrangea* (Hydrangeaceae), and *Eucnide* (Loasaceae) suggests that at least two reversals are necessary to generate polypetally in these taxa from putatively sympetalous ancestors. Alternatively, these taxa may represent the retention of the ancestral state of polypetally with sympetally arising at least four times during evolution of the Asteridae.

Phylogenetic Relationships Within the Asteridae. Below we discuss the ten major groups of Asteridae sens. lat. seen in the cladogram (Fig. 2). Our results, although preliminary, are generally consistent with traditional morphological groupings and with the results obtained from *rbc*L sequence data (Olmstead et al., 1992). The results of our analysis regarding representatives of the Rosidae (Apiaceae, Araliaceae, Cornaceae, Hydrangeaceae) and Dilleniidae (Fouquieriaceae, Loasaceae) sensu Cronquist in the Asteridae sens. lat. suggest that neither of these subclasses are monophyletic and that their higher-level relationships are in need of further study.

(A) *Hydrangeaceae, Cornaceae, and Loasaceae.* The traditional association of Hydrangeaceae with the Saxifragaceae and its allied woody families has been disputed by Dahlgren (1980, 1983), Takhtajan (1987), and Soltis et al. (1990b). Dahlgren considered the Hydrangeaceae to be closely allied to Cornaceae and treated them along with the Caprifoliaceae in his Cornales, an order he described as closely related to the Dipsacales, Fouquieriales, and Ericales. On the basis of morphological and chemical data, Hufford (1992) showed that the "woody saxifrages" (e.g., Hydrangeaceae) are more closely related to members of the Cornaceae and Loasaceae than to the "herbaceous saxifrages" (e.g., Saxifragaceae sens. str.). Our results indicate that *Hydrangea*, *Cornus*, and *Eucnide* (Loasaceae) belong to a monophyletic group, with the relationships among the genera unresolved.

Historically, the affinities of the Loasaceae have been obscure. Takhtajan (1969) initially considered the Loasaceae to be related to the Boraginaceae and Hydrophyllaceae. Subsequent treatments either placed Loasaceae in a separate order, Loasales, related to the Dipsacales and the Polemoniales (Takhtajan, 1980), or to the Gentianales (Takhtajan, 1987). Hufford (1990, 1992) provided evidence against the hypothesis that the Loasaceae share a common ancestry with members of the Dilleniidae, a position favored by Cronquist (1981), and instead supported Dahlgren's (1983) suggestion that the family shares an ancestry with the Dipsacales, Cornales, and the "woody saxifrages." On the basis of wood anatomy, Carlquist (1992) believes that Loasales are not far from such orders as the Dipsacales or Cornales. The results presented here support, in part, the hypotheses presented by Hufford, Dahlgren, and Carlquist, attesting to a common ancestry for Loasaceae and Cornales.

The systematic position of *Aucuba* is unclear. This genus is placed in the Cornaceae by Cronquist (1981), whereas Takhtajan (1980) placed it in the closely related family Aucubaceae. *Aucuba* differs from all other cornaceous taxa in its chemistry,

floral anatomy, embryology, and pollen structure (Rodriguez, 1971; Takhtajan, 1980). Our results place *Aucuba* outside of Group A and suggest that if the Cornaceae are circumscribed to include this taxon, the family would be polyphyletic. However, by imposing the constraint on the cladistic analysis that *Aucuba* and *Cornus* species form a monophyletic group, shortest trees one step longer than the most parsimonious trees were found. Consequently, the putative polyphyly exhibited by this group must be regarded as tentative.

(B) *Solanales.* The clade designated as Solanales in this analysis comprises three families (sensu Cronquist): Solanaceae, Nolanaceae, and Convolvulaceae. It is widely agreed that the Solanaceae and Nolanaceae are closely related. In the past, Nolanaceae have either been treated at the subfamilial level within the Solanaceae (D'Arcy, 1979) or as a closely related segregate family derived from the Solanaceae (Cronquist, 1981; Takhtajan, 1987). Our results are consistent with those of Thorne (1968), D'Arcy (1979), and Olmstead & Palmer (1992), affirming the submersion of *Nolana* within the Solanaceae. The position of the morphologically distinct *Schizanthus* as the earliest diverging lineage in the Solanaceae is also supported by the more extensive restriction site analysis of Olmstead & Palmer (1992). Our results differ from those of Olmstead & Palmer in the relationships among *Nicotiana, Nolana,* and *Solandra*. Our results place *Nicotiana* and *Nolana* as sister taxa, with *Solandra* basal to this group (Fig. 2). Olmstead & Palmer show that *Nolana* and *Solandra* are more closely related to each other than either is to *Nicotiana.*

The Solanaceae and Convolvulaceae emerge as sister groups in our analysis. Anatomical and phytochemical similarities between these two families have suggested a close relationship in the past (Cronquist, 1981; Thorne, 1983). Moreover, a phylogenetic analysis of *rbc*L sequence data (Olmstead et al., 1992) corroborates this relationship.

(C) *Boraginales.* The clade designated as the Boraginales is represented by two families in this analysis: Boraginaceae and Hydrophyllaceae. The close morphological relationship between these families has long been evident (e.g., Dahlgren, 1983; Takhtajan, 1987), in spite of Cronquist's (1981) treatment to the contrary, wherein the Boraginaceae are placed alongside the Lamiaceae and Verbenaceae in his Lamiales, and the Hydrophyllaceae are placed in his Solanales. Cronquist does, however, acknowledge the strong similarity between them. The division of this clade into two distinct lineages, with *Heliotropium* (Boraginaceae) placed in a clade with *Hydrophyllum* and *Eriodictyon* (Hydrophyllaceae), raises questions about the circumscription and generic relationships of the two families.

(D) *Scrophulariales plus Lamiales.* The largest group recognized in the analysis is composed of families that have traditionally been considered in the Scrophulariales and Lamiales. They are treated here together, owing to the lack of resolution within the clade. Included here are representatives from 12 families, Scrophulariaceae, Globulariaceae, Gesneriaceae, Acanthaceae, Pedaliaceae, Bignoniaceae, Myoporaceae, Lamiaceae, Verbenaceae, Callitrichaceae, Buddlejaceae, and Oleaceae. It is widely agreed that the first seven families are closely related. The Lamiaceae and Verbenaceae form a closely related pair (Cantino, 1992) and are often treated in the separate order Lamiales; however, based on our data, the distinction between this order and the Scrophulariales is weak. The association of Lamiaceae and Verbenaceae with the Scrophulariales suggested by cpDNA restriction site data (Fig. 2) and *rbc*L sequence data (Olmstead et al., 1992) is in accordance with several previous treatments (Wagenitz, 1977; Cantino, 1982; Dahlgren, 1983), but disagrees with hypotheses by Takhtajan (1980) and Cronquist (1981), who suggested that the Boraginales are the extant group most closely related to these two families.

Our results further suggest that Buddlejaceae, frequently regarded as a tribe in the Loganiaceae (Gentianales), are misplaced and should be considered within the Scrophulariales plus Lamiales. Callitrichaceae, often included either in the Lamiales (Dahlgren, 1983; Thorne, 1983; Takhtajan, 1987) or in their own order near the Scrophulariales (Cronquist, 1981), lie within the Scrophulariales plus Lamiales clade. These results are consistent with those of *rbc*L sequence analysis (Olmstead et al., 1992, and unpublished).

The affinities of the Oleaceae have also been disputed. The family has been placed either in its own order (Oleales) near the Gentianales (Dahlgren, 1983; Thorne, 1983; Takhtajan, 1987), within the Gentianales (Stebbins, 1974), or in the Scrophulariales (Cronquist, 1981). Disparities in floral symmetry, phytochemistry, embryology, and anatomy between the Oleaceae and either the Gentianales or the Scrophulariales have precluded a satisfactory placement in either order. Our results suggest that the Oleaceae (as represented by *Forsythia, Ligustrum,* and *Syringa*) are more closely

related to the Scrophulariales than to the Gentianales. This is consistent with the *rbc*L analysis of Olmstead et al. (1992), which placed the family in a basal position in the Scrophulariales.

Of the seven scrophularialean families for which more than one species was examined, only two, Acanthaceae and Myoporaceae, emerge as monophyletic. The gynobasic-styled Lamiaceae (*Stachys*, *Prasium*, *Comanthosphace*, and *Pogostemon*) also emerge as distinct in agreement with Cantino (1992), but their relationship with other mints and the Verbenaceae is not resolved.

The lack of resolution within and among the families that constitute the Scrophulariales is likely due to two factors. First, the low number of informative restriction sites within these taxa presents a problem. Of the 55 phylogenetically informative characters used in the cladistic analysis, only 10 provide information on relationships among the 22 species from nine families that make up the Scrophulariales (Table 1). Several of these DNAs are identical in all 77 restriction sites compared. The estimated pairwise percent sequence divergence (Brown et al., 1979) between *Syringa* (Oleaceae) and *Verbascum* (Scrophulariaceae), which may represent extremes within the order (see above), is 0.7% (Table 3). Data from only four restriction enzymes were available; as data from more restriction enzymes and from gene sequencing are included in subsequent phylogenetic analyses, greater resolution among the families is expected. A study of this nature is currently in progress (C. Morden, C. dePamphilis & J. Palmer, unpublished).

Second, the Scrophulariales are a relatively homogeneous order, with the morphological similarities among its constituent families emphasized by many. Several families are connected by genera thought to be transitional or of uncertain placement (Armstrong, 1985); consequently, precise circumscriptions of some families are ambiguous. Moreover, some members of the order are considered to be specialized derivatives of the Scrophulariaceae, the largest and putatively central family in the order (Cronquist, 1981). Cladistic analyses using primarily morphological characters (Lu, 1990) and *rbc*L sequence data (Olmstead et al., 1992) offered little resolution among the families comprising the orders Scrophulariales and Lamiales. Diversification of the Scrophulariales into families and perhaps even genera may have occurred rapidly relative to other orders within the subclass.

(E) *Gentianales.* The Apocynaceae, Asclepiadaceae, Loganiaceae, Gentianaceae, and Rubiaceae are representatives of the Gentianales in this study. Reasonable consensus exists among systematists regarding the circumscription and, to a lesser degree, the infraordinal structure of the Gentianales. The first four families listed above are closely related in all modern taxonomic systems and are relatively homogeneous in wood anatomy (Carlquist, 1992), morphology, and phytochemistry. The Menyanthaceae, Buddlejaceae, and Oleaceae, often included in the Gentianales, are placed elsewhere in our analysis (see Groups D and G).

Although most systematists treat the Apocynaceae and Asclepiadaceae as distinct families, some believe that because few characters clearly differentiate these taxa, it would be more appropriate to treat them as a single family (Hallier, 1905; Demeter, 1922; Stebbins, 1974; Thorne, 1983). Our results support the latter view. The Apocynaceae form a monophyletic group when the Asclepiadaceae (represented by *Asclepias* and *Periploca*) are included within it (Fig. 2).

The Loganiaceae are a morphologically heterogeneous group, with many segregate families recognized by some authors. The family is represented here by three genera: *Fagraea*, *Spigelia*, and *Strychnos*. The latter two genera form a clade in our analysis, whereas *Fagraea* is grouped with the Gentianaceae, suggesting that it might be misplaced in the Loganiaceae (see Jensen, 1992). Alternatively, the Loganiaceae may be a paraphyletic group ancestral to the Apocynaceae and Gentianaceae.

The Rubiaceae, represented here by *Pentas*, are often associated with the Gentianales (Bremer, 1992). In the treatments of Takhtajan (1980, 1987), Thorne (1983), Dahlgren (1983), and Wagenitz (1977), the Rubiaceae are included in the Gentianales. However, their lack of internal phloem (otherwise ubiquitous in the order) and the presence of an inferior ovary (otherwise superior with few exceptions) make the family stand apart from the rest of the order. Cronquist (1981) excluded the Rubiaceae from the Gentianales and considered them in their own order, the Rubiales. Similarly, wood anatomy suggests that the Rubiaceae may not belong within the Gentianales (Carlquist, 1992), but rather in a neighboring monofamilial order. Our data place the Rubiaceae as the most basal branch within the Gentianales (Fig. 2), and are, therefore, consistent with either their inclusion in the Gentianales or their segregation in a separate order.

(F) *Viburnum and Sambucus.* The relationships between *Viburnum* and *Sambucus*, and between these two taxa and other genera traditionally

placed within the Caprifoliaceae, have been subject to much speculation. Our data support the close relationship between *Viburnum* and *Sambucus* posited by Donoghue (1983) on the basis of morphological evidence. The separation of *Viburnum* and *Sambucus* (plus *Adoxa*) from the rest of the Caprifoliaceae and from other Dipsacales has been suggested by Donoghue and coworkers on the basis of morphological evidence, *rbc*L sequence data, and preliminary cpDNA restriction site data (Donoghue, 1983, 1990; Donoghue et al., 1992). Our data also agree with this view. The absence of two restriction fragment length variants in *Viburnum* and *Sambucus*, otherwise present in the four other Caprifoliaceae examined (Table 2), further distinguishes them from the Caprifoliaceae sens. str. but is neutral with respect to the controversy concerning their phylogenetic affinities.

The putative sister-group relationship between the clade consisting of *Viburnum* and *Sambucus* and the Asterales, as seen in Figure 2, is complicated by the fact that more than one most parsimonious reconstruction (MPR) exists (Swofford & Maddison, 1987). Because the branch uniting Groups F and G has zero-length under one MPR but a length of one under a different MPR, PAUP retains the zero-length branch (Swofford, 1990). The collapse of this zero-length branch yields an unresolved trichotomy consisting of Groups A through E as one branch, Group G as another branch, and Group F as the third branch (Fig. 3). Given this, the close relationship of *Viburnum* and *Sambucus* to the Asterales in Figure 2 must be regarded as tentative. On the basis of *rbc*L sequence data (Donoghue et al., 1992), the connection between *Viburnum* and *Sambucus* and the Asterales is also unclear.

(G) *Asterales sensu lato.* The clade identified as the Asterales in Figure 2 comprises representatives from the Asteraceae, Calyceraceae, and Menyanthaceae. Material from the allied Campanulales (Goodeniaceae and Campanulaceae) was examined (Table 1), but extensive length and sequence variation in their cpDNAs made comparative mapping and the confirmation of homology of restriction sites impossible. Therefore, these families were not included in the phylogenetic analysis. Sister-group relationships between *Gamocarpha* and *Boopis* (Calyceraceae), and between *Villarsia* and *Nymphoides* (Menyanthaceae), are evident; however, relationships among the Asteraceae, Calyceraceae, and Menyanthaceae are not resolved. Other than the loss of the *rpl*2 intron in the Menyanthaceae (Table 3; Downie et al., 1991), the DNAs of *Menyanthes* and *Fauria* (Menyanthaceae) and *Barnadesia* (Asteraceae) are identical at all restriction fragments compared, with only two character differences separating *Barnadesia* from *Lactuca* (Fig. 3). A closer relationship between *Villarsia* and *Nymphoides* than between these two genera and any other in the Menyanthaceae is also inferred from morphological and flavonoid chemical data (Ornduff, 1973; Bohm et al., 1986).

Affinities between the Asteraceae and Calyceraceae have often been claimed based on floral morphology and wood anatomy (Turner, 1977; Skvarla et al., 1977; Carlquist, 1992). Dahlgren (1983) and Thorne (1983) placed the Calyceraceae in the Dipsacales, whereas Cronquist (1981) placed them in their own order, the Calycerales, which he stated is related to and probably derived from the Dipsacales. Our results strongly indicate that the Calyceraceae belong within the clade designated here as the Asterales and not within the Dipsacales. Phylogenetic analyses of *rbc*L sequence data corroborate these results and indicate further that the families Calyceraceae and Goodeniaceae are the closest living relatives to the Asteraceae (Michaels et al., in prep.; Olmstead et al., 1992).

The presence of Menyanthaceae in the asteralean clade is unexpected. Once relegated to infrafamilial status within the Gentianaceae (Bentham, 1876; Rendle, 1925), the Menyanthaceae are now recognized at the familial level. Discordance between anatomical and chemical characters has precluded a consensus on its ordinal placement. Most modern systematists include the Menyanthaceae within the Gentianales (e.g., Takhtajan, 1987), but Cronquist (1981) viewed their position here as "discordant" and placed them in the Solanales. Our data are in agreement with the strong evidence from *rbc*L sequences (Olmstead et al., 1992) that instead places Menyanthaceae within the Asterales. Although the families differ strikingly in flower and inflorescence morphology, a close relationship between the Menyanthaceae and Asteraceae was posited by Yamazaki (1971) on the basis of similarity in embryo development, and by Pollard & Amuti (1981) on the basis of common possession of inulin.

(H) *Apiales.* Cronquist (1981) placed the Apiales (Apiaceae and Araliaceae) in the subclass Rosidae. The morphological resemblance among the Apiales, Cornaceae, and some genera of Caprifoliaceae has been noted (Thorne, 1983), with the Apiales considered allied to the Cornales and placed in the Rosidae (Cronquist, 1981). Our results agree that the Araliaceae and Apiaceae are closely related

(e.g., Takhtajan, 1980; Cronquist, 1981; Thorne, 1983). The placement of the Apiales as sister group to the Dipsacales in our study is variously supported by *rbc*L sequence data. Our data are in close agreement with Olmstead et al. (1992) in placing the Apiales as sister group to the Dipsacales, but differ from Donoghue et al. (1992) in which the position of the Apiales in relation to the Dipsacales (and Asterales) is uncertain.

(I) *Dipsacales.* Included here are taxa belonging to the Caprifoliaceae, Dipsacaceae, and Valerianaceae. *Viburnum* and *Sambucus* are excluded from this clade in our analysis (see above discussion of Group F). The Dipsacaceae emerge as monophyletic and are nested along with Valerianaceae within a paraphyletic Caprifoliaceae (Fig. 2). *Kolkwitzia*, belonging to the tribe Linnaeeae of Caprifoliaceae, and *Valeriana* (Valerianaceae) emerge as sister taxa in our analysis. A close relationship has been proposed between this tribe and the Valerianaceae (Wilkinson, 1949; Donoghue, 1983; Donoghue et al., 1992). *Lonicera* and *Symphoricarpos* also emerge as more closely related to each other than either is to any other member of the Caprifoliaceae. A more detailed analysis of the phylogeny of the Dipsacales based on *rbc*L sequences is presented elsewhere in this volume (Donoghue et al., 1992).

(J) *Fouquieria and Polemoniaceae.* The systematic positions of the Fouquieriaceae and the Polemoniaceae have long been matters of controversy. The Fouquieriaceae have been variously placed in the Violales (Dilleniidae; Cronquist, 1981), the Tamaricales (Dilleniidae; Takhtajan, 1980), or in their own order, the Fouquieriales (Corniflorae, near Ericales and Cornales; Dahlgren, 1983). The Polemoniaceae, once thought to be allied with the Caryophyllales (Caryophyllidae) or with the Geraniales (Rosidae; see review in Dawson, 1936), are often placed in the Asteridae (Cronquist, 1981; Takhtajan, 1980). Henrickson (1967) and Thorne (1977) considered the Polemoniaceae to have affinities with the Fouquieriaceae. Hufford (1992) allied the Polemoniaceae with the Pittosporaceae and treated this clade as sister group to the Asteridae, and placed the Fouquieriaceae as the sister group to the Ericales (Dilleniidae). The latter hypothesis is supported by Olmstead et al. (1992, and unpublished), whose results showed that the Fouquieriaceae and Polemoniaceae are part of a monophyletic Ericales. The placement of the Fouquieriaceae alongside the Polemoniaceae in this study is in agreement with Henrickson's and Thorne's earlier hypotheses: however, their close relationship is not well supported.

Fouquieria possesses a number of distinctive features that make it equally as anomalous in the Dilleniidae as in the Asteridae (Wagenitz, 1977; Cronquist, 1981; Hufford, 1992). The basal position of *Fouquieria* and Polemoniaceae in Figures 2 and 3 is surprising and demands further attention. In this regard, it is noteworthy that some support for this position is also found in a *rbc*L sequence phylogeny (Olmstead et al., 1992, and unpublished).

Conclusions

Comparative restriction site mapping of IR sequences of chloroplast genomes from 99 species and 37 families of Asteridae and putatively allied taxa in the Rosidae and Dilleniidae allows for phylogenetic inference at high taxonomic levels. This study demonstrates for the first time the potential of this approach for illuminating phylogenetic relationships at the familial and ordinal level. Wolfe et al. (1987) have demonstrated that rates of nucleotide substitutions at silent sites and in noncoding sequences in the IR are 4–6 times lower than those in single-copy regions. Consequently, by focusing exclusively on the highly conserved IR region of the chloroplast genome one can predict that comparative restriction site mapping studies can be extended to evolutionary depths 4–6 times greater than that to which they have been applied previously. Since restriction site variation within the entire chloroplast genome has been used successfully to infer phylogenies for a few large families, it is not surprising that restriction site mapping of the IR works well over the whole Asteridae.

This conservatism in IR restriction site mutations is, however, both a blessing and a curse. At appropriate levels of nucleotide divergence, these data can be used in a cladistic analysis to infer relationships; however, the extreme conservatism of the IR precludes robust hypotheses of relationships among relatively closely related taxa. Also, excessive divergence in restriction sites in several families (Table 1), particularly those that have lost one entire segment of the cpDNA IR, prevents alignment of restriction maps and assessment of site homology. Divergence at the structural level, whether due to inversions or major length variation, also limits the utility of this approach. At deep phylogenetic levels, the approach presented here will yield fewer informative characters than DNA sequencing but is clearly a useful adjunct approach

to sequencing. The utility of *rbc*L sequence data in inferring relationships across different subclasses of angiosperms has been demonstrated by Olmstead et al. (1992).

Phylogenetic analysis of cpDNA IR restriction site variation provides a means of reassessing the traditional and largely morphologically based classifications of the Asteridae. The results presented here are preliminary in the sense that the ratio of informative characters to taxa is low and therefore are not a sufficient basis for a new classification of the subclass. Nevertheless, they provide a set of explicit hypotheses about relationships in the Asteridae that can be tested as additional evidence becomes available. These data provide important corroborating evidence for other contemporary studies focusing on cpDNA sequence data (Donoghue et al., 1992; Michaels et al., in prep.; Olmstead et al., 1992). If subsequent analyses support the results presented here, some realignments in the circumscription and classification of the Asteridae will be in order.

The following general conclusions are reached concerning phylogenetic relationships in Asteridae sensu lato. (1) Six distinct clades that broadly correspond to traditionally recognized orders in the Asteridae can be circumscribed: Solanales, Boraginales, Scrophulariales plus Lamiales, Gentianales, Asterales sens. lat. (including Calyceraceae and Menyanthaceae), and Dipsacales (minus *Viburnum* and *Sambucus*). Infraordinal relationships are reasonably well resolved for four of these orders, but not for the Scrophulariales plus Lamiales and Asterales sens. lat. Interordinal relationships remain poorly resolved. (2) The Apiales, included here in the Asteridae sens. lat., may be the sister group to the Dipsacales. Members of Hydrangeaceae, Cornaceae, and Loasaceae emerge as closely allied and also fall within the Asteridae sens. lat. Consequently, the Asteridae as traditionally circumscribed do not form a monophyletic group. (3) The Caprifoliaceae, in any traditional sense, cannot be monophyletic. *Viburnum* and *Sambucus* emerge as a distinct clade, well separated from the four other genera of Caprifoliaceae examined. Dipsacaceae and Valerianaceae arise from within the Caprifoliceae sens. str., with *Kolkwitzia* as a sister group to Valerianaceae. The Caprifoliaceae are, at best, paraphyletic. (4) Some light is shed on the placement of several problematic taxa. Menyanthaceae are placed in the Asterales sens. lat., Buddlejaceae and Callitrichaceae are placed within the Scrophulariales plus Lamiales, *Nolana* is placed within the Solanaceae, and the Rubiaceae are allied to the Gentianales. (5) Asclepiadaceae are derived from Apocynaceae.

Of the 25 families for which more than one species was examined (Table 1), ten (Acanthaceae, Apocynaceae sens. lat., Araliaceae, Asclepiadaceae, Calyceraceae, Convolvulaceae, Dipsacaceae, Myoporaceae, Polemoniaceae, and Solanaceae) constitute monophyletic groups; two (Caprifoliaceae sens. lat. and Cornaceae) may be polyphyletic; two may be paraphyletic (Apocynaceae and Caprifoliaceae sens. str.); and 12 are unresolved with the data at hand.

The results presented here are generally consistent with traditional morphological groupings and are highly congruent with a phylogenetic analysis of *rbc*L sequence data (Olmstead et al., 1992). The lack of resolution in many portions of the consensus tree is primarily due to an insufficient number of characters rather than conflict among characters, for many of the branches are supported by only one character change (Fig. 3). Future analyses should therefore benefit from the use of additional restriction enzymes to increase the number of restriction sites sampled. With more characters, measures of statistical evaluation (e.g., bootstrap sampling) can be applied. Other problematic families, such as the Plantaginaceae, Lentibulariaceae, and Retziaceae, can be included in further analyses.

Phylogenetic relationships based on these molecular data should help to assess the relative importance of traditional characters (e.g., morphological, phytochemical, embryological) currently used in circumscribing orders and families in the Asteridae. The occurrence of unexpected relationships suggests that a reevaluation of characters is in order, particularly in the taxa heretofore included in the Rosidae and Dilleniidae. In the future, the approach presented here should complement DNA sequencing and structural rearrangement studies in elucidating relationships at higher taxonomic levels.

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